



Chemical Composition, Antioxidant and Antibacterial Activity of *Bunium persicum*, *Eucalyptus globulus*, and Rose Water on Multidrug-Resistant *Listeria* Species

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Abstract

This research was aimed at investigating the antioxidant and antibacterial activity of *Bunium persicum*, *Eucalyptus globulus*, and rose water on multidrug-resistant *Listeria* species. The antibiotic resistance of *Listeria* spp obtained from seafood samples were determined by the Kirby-Bauer method. The antioxidant and antibacterial activity of the essential oils and extracts were evaluated using ferric reducing antioxidant power and microdilution methods, respectively. A total 2 samples (1.88%) were positive for *Listeria* spp. *L. monocytogenes* was found to be resistant to ampicillin, amoxicillin/clavulanic acid, penicillin, vancomycin, and kanamycin. *B. persicum* essential oil showed the greatest antioxidant activity ($248.56 \pm 1.09 \mu\text{M Fe}^{2+}/\text{g}$). The *E. globulus* essential oil showed consistently strong antimicrobial activity against *L. monocytogenes* and *L. grayi*, while rose water showed no antimicrobial activity against any of the tested bacterial strains. The results showed that after adding the *B. persicum* and *E. globulus* essential oils to bacteria, the cell components' release increased significantly.

Keywords

antibacterial activity, multidrug resistance, seafood, *Listeria*

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Food-borne pathogenic bacteria have been considered as the main causes of food-borne infections in worldwide.^{1,2} Among food pathogens, *Listeria monocytogenes* is the only species of the genus *Listeria* that has been involved in known food-borne outbreaks of listeriosis and causing 10% to 40% mortality in patients.^{1,3,4} *L. monocytogenes* is ubiquitous and can grow in high salt concentrations (10%), at a wide range of temperatures (1°C-45°C), at pH 4.6 to 9.6 and can be found in raw products of animal origin and fresh vegetables.⁵ Also, the contamination could happen in processed food through contaminated waters and environments as well as during transportation.

The Centers for Disease Control and Prevention⁶ has reported human listeriosis ascribing to consumption of contaminated cantaloupe in the United States. Also, numerous studies in Iran have detected *L. monocytogenes* and its other species in various types of foods, including seafood,⁷ open-air fish markets,⁸ raw milk,⁹ meat and meat products, and ready-to-eat foods.^{10,11}

Nowadays, the development of antibiotic resistance both in animal and human zoonotic bacterial pathogens has been

related to the administration of antimicrobial agents in food animal production as growth promoters and their extensive therapeutic use. The antimicrobial agents can be passed through the food chain and contribute to the emergence of resistant bacteria that can be transferred directly to humans after consumption.^{12,13} Previous studies have reported the multidrug-resistant *Listeria* spp isolated from raw milk, milking equipment, dairy workers,¹⁴ ready-to-eat products of animal origin,¹⁵ and fresh and smoked fish.¹⁶

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Therefore, there is an urgent need for control the spread of multidrug-resistant pathogens to new antimicrobial agents as a strategy. Presently, extracts and essential oils of medicinal plants are used as a novel source of effective antimicrobial agents.¹⁷ Previous works have demonstrated the antimicrobial properties of many plant essential oils and extracts as natural substances.^{1,2,17,18} The presence of bioactive compounds such as phenolic substances in extracts and essential oils may react with microorganisms and restrain microbial growth.¹⁹ Therefore, the aim of this study was to investigate the chemical composition, antioxidant and antibacterial activity of *Bunium persicum*, *Eucalyptus globulus*, and rose water on multidrug-resistant *Listeria* species.

Materials and Methods

Sample Collection

The minimum sample size required was estimated based on the single proportion formula:

$$n = \frac{Z^2 pq}{d^2}$$

where n is the minimum sample size required, Z is the reliability coefficient at 95% confidence interval (1.96), p is the prevalence estimate, q is equal to $1 - p$, and d is the acceptable error (0.05). According to a previously published prevalence estimate of 7% reported from a study on *Listeria* species in fresh and frozen fish and shrimp in Iran.²⁰

Accordingly, a total of 106 raw samples of various seafood including shrimp ($n = 40$) and fresh fish ($n = 66$) were purchased from randomly selected retail markets located in Kashan, Iran from March 2016 to June 2017. All samples were immediately transferred to the food microbiology laboratory, Kashan University of Medical Sciences, in portable insulated cold-boxes. The samples were analyzed on the day they were collected.

Isolation and Identification of *Listeria* spp

A 25-g portion of each food sample was added to 225 mL of *Listeria* enrichment broth (HiMedia Laboratories Limited, Mumbai, India) and stomached for 3 minutes and incubated at 37°C for 48 hours. Then, a loopful of each enrichment culture was spread plated onto *Listeria* Selective Agar (HiMedia Laboratories Pvt Ltd, India) and was incubated for 24 hours, at 37°C. Following incubation, the suspected colonies with a dark brown color or black halo were selected and transferred onto tryptic soy agar (Merck Co, Darmstadt, Germany) and incubated for 24 hours, at 37°C. The isolates were identified using conventional methods: Gram staining, catalase, typical umbrella motility and fermentation of mannitol, rhamnose, and xylose.²¹

Antimicrobial Susceptibility

Antimicrobial susceptibility testing of the *Listeria* isolates was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (Merck Co, Darmstadt, Germany) according to the Clinical and Laboratory Standards Institute.²² A bacterial suspension with equivalent turbidity to 0.5 McFarland standard (1.5×10^8 cfu/mL) was prepared in sterile phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). The sterile swab stick was

dipped into bacterial suspension and then on the surface of agar was uniformly inoculated. Five antibiotic disks were placed for each plate and incubated at 35°C for 24 hours. Inhibition zones on agar plate were measured after 24 hours and the results were recorded in accordance with interpretive criteria provided by the Clinical and Laboratory Standards Institute. The antibiotics discs (HiMedia Laboratories Pvt Ltd, Mumbai, India) are reported as follows: ampicillin (10 µg), amoxicillin/clavulanic acid, clindamycin (2 µg), erythromycin (15 µg), gentamycin (10 µg), ciprofloxacin (5 µg), penicillin (10 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole (10 µg), vancomycin (30 µg), nitrofurantoin (300 µg), norfloxacin (30 µg), kanamycin (30 µg), ceftriaxone (30 µg), and chloramphenicol (30 µg).

Plant Material and Preparation of the Essential Oils and Extracts

Samples of *B. persicum*, *E. globulus*, and rose water were purchased from a reputable grocery in Kashan, Iran. The collected samples were identified and stored at the herbarium of the Research Center for Biochemistry and Nutrition in Metabolic Diseases of Kashan University of Medical Sciences, Iran (Nos. 1b, 2e, and 3r).

To prepare essential oils, the 100 g dried plants were hydrodistilled for 4 hours using a Clevenger type apparatus. The essential oils were then dehydrated over anhydrous sodium sulfate and kept in sealed vials at 4°C.

To prepare ethanolic extracts, 100 g of the dried powder of the plants were mixed with 500 cm³ of 80% ethanol and kept at room temperature (22°C) for 24 hours. The obtained extracts were filtered by filter paper and entered into rotary device (to remove solvent). The obtained alcoholic extracts were dried at the temperature of 40°C in an incubator.¹⁸ For the antibacterial properties, several dilutions of the essential oils and extracts were done using 5% (v/v) aqueous dimethyl sulfoxide (DMSO; Merck Co, Darmstadt, Germany) and sterilized by filtration through a 0.45-µm membrane filter.

Essential Oils and Extracts Analysis

The gas chromatographic analyses of essential oils were carried out by an Agilent 6890 GC system with a HP-5MS (60 m × 0.25 mm, film thickness 0.25 µm). The carrier gas (helium) was used at a flow rate of 1.0 mL/min. The gas chromatograph oven temperature was kept at 40°C for 1 minute and programmed to 230°C at the rate of 3°C/min. The injector and detector temperatures were 230°C and 250°C, respectively. Quantitative data were obtained electronically from flame ionization detector area percent data. The main chemical constituents of essential oils were compared with analytical standard and tested in triplicate.

The amount of total phenolic content was measured in ethanolic extracts by colorimetric method using Folin-Ciocalteu. Standard solutions with concentration of 12.5, 25, 50, 62.5, 100, and 125 ppm were prepared from gallic acid in 60% solution. A total of 0.1 mL of each solution was transferred to a test tube and 0.5 mL of reagent Folin-Ciocalteu solution 10% was added and after 3 to 8 minutes, 0.4 mL 7.5% sodium carbonate solution was added to it, then the tubes were kept at laboratory temperature for 30 minutes. The optical absorption was measured at a wavelength of 765 nm by a spectrophotometer (Unico UV-2100, Dayton, NJ, USA) and a standard curve was prepared. Then 0.01 to 0.02 g of the dried extract was dissolved in 60% methanol up to the volume of 10 mL. Total phenol content was

determined based on Folin-Ciocalteu method with the difference that instead of the standard solution, 0.1 mL of the extract solution was added. The obtained absorbance rate was posed at the standard curve and thus the total phenol content of the extract in mg/g gallic acid equivalent was estimated.²³

Ferric Reducing Antioxidant Power Assay

The determination of the total antioxidant activity of plant extracts and essential oils was done by iron reduction (ferric reducing antioxidant power assay).²⁴ The stock solutions included 300 μ M acetate buffer pH 3.6 (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$), 10 μ M 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 μ M HCl, and 20 μ M $FeCl_3 \cdot 6H_2O$ solution. FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution. The temperature of the solution was raised to 37°C before use. A volume of 200 μ L of the plant extracts (1 mg/mL) and essential oils was mixed with 2800 μ L of the ferric reducing antioxidant power reagent. The absorbance was measured after 30-minute incubation at 37°C in dark condition at 593 nm. The values were calculated from a calibration curve obtained with $FeSO_4 \cdot 7H_2O$ (100-1000 μ M). Final results were expressed as μ M Fe^{2+} /g extract or essential oil.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Assays

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The 96-well plastic microdilution tray were prepared by dispensing into each well 95 μ L of Mueller-Hinton broth (MHB; Merck Co, Darmstadt, Germany) and 5 μ L of each isolate suspension equivalent to 0.5 McFarland. Finally, 100 μ L of consecutive dilution of (serial 2-fold dilutions) each extract or essential oil were added to each well. Positive and negative controls were considered as follows: positive control—195 μ L MHB containing DMSO and 5 μ L of bacterial suspension without extracts and essential oils and negative control—200 μ L of MHB containing DMSO without bacterial inoculum. After mixing the samples by shaker (with the rate of 300 rpm per 20 S), they were placed in an incubator for 18 to 24 hours at 37°C. The wells were examined according to the presence or lack of turbidity. Dilution plate of the well containing the lowest concentration of plant extracts or essential oils that inhibited growth of bacteria (lack of turbidity) was determined as the MIC. Furthermore, the lowest concentration that showed no visible growth on Mueller-Hinton agar was determined as the MBC.²⁵

Integrity of Cell Membrane

The cell integrity of *Listeria* strains is tested by determining the release of cell components into supernatant according to the method described by Xu et al²⁶ with some modifications. Bacteria from the working culture of *Listeria* strains were collected by centrifuged for 15 minutes at 5000 rpm, washed 3 times, and resuspended in 0.1 M phosphate buffered solution. Then, cell suspension obtained were incubated at 37°C under agitation for 4 hours in the presence of essential oils and extracts at MIC concentration. After that, 10 mL of samples were collected and centrifuged at 11 000 rpm for 3 minutes. The concentration of the released components including especially of

Table 1. Antimicrobial Resistance of *Listeria* spp Isolated From Seafood in Kashan, Iran.

Antimicrobials	<i>Listeria monocytogenes</i>	<i>Listeria grayi</i>
Ampicillin (10 μ g)	R	R
Amoxicillin/clavulanic acid	R	R
Clindamycin (2 μ g)	I	R
Erythromycin (15 μ g)	I	R
Methicillin (5 μ g)	R	R
Gentamycin (10 μ g)	S	R
Ciprofloxacin (5 μ g)	S	S
Penicillin (10 μ g)	R	R
Tetracycline (30 μ g)	I	R
Trimethoprim/Sulfamethoxazole (10 μ g)	S	S
Vancomycin (30 μ g)	R	R
Nitrofurantoin (300 μ g)	I	R
Norfloxacin (30 μ g)	S	R
Kanamycin (30 μ g)	R	R
Ceftriaxone (30 μ g)	I	R
Chloramphenicol (30 μ g)	S	R

Abbreviations: R, resistant; I, intermediate; S, susceptible.

nucleic acids, 1 mL supernatant was used to measure ultraviolet absorption at 260 nm.

Statistical Analysis

Data were analyzed using SPSS version 18.0 (IBM Corp, Armonk, NY, USA) and descriptive statistic was used. The results of all experiments were expressed as the mean \pm standard deviation of triplicates. Analysis of variance was carried out to determine significant differences ($P < .05$) between means.

Results

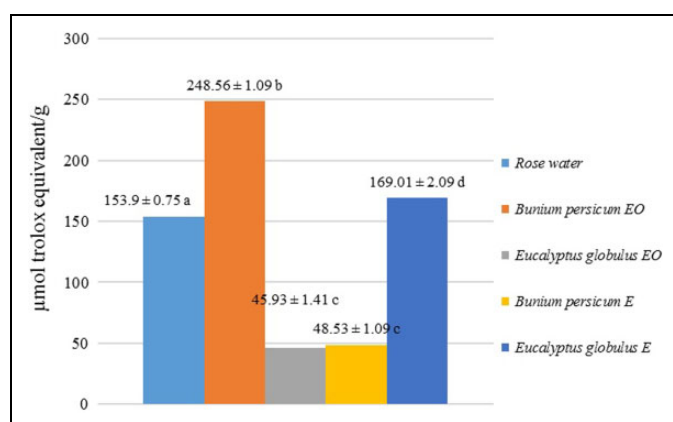
In the present study, using cultural techniques, 2 of 106 samples (1.88%) were positive for *Listeria* strains. No *Listeria* was isolated from fresh fish samples. In contrast, 2 shrimp (5%) samples were contaminated with *L. monocytogenes* (2.5%) and *L. grayi* (2.5%). The resistance pattern of *Listeria* spp isolates to 16 antibiotics tested in this study is shown in Table 1. The 2 isolates (100%) were multidrug resistant. In addition, 2 isolates were susceptible to trimethoprim/sulfamethoxazole and ciprofloxacin. The *L. monocytogenes* was more sensitive to antibiotics than *L. grayi*.

The main chemical composition of the essential oils is presented in Table 2. The total phenolic content of *B. persicum* and *E. globulus* extracts were 4.32 ± 0.9 and 23.06 ± 1.4 mg/g gallic acid equivalent, respectively.

The total antioxidant activity of the 2 essential oils, 2 extracts, and rose water was measured by FRAP assay (Figure 1). *B. persicum* essential oil showed the greatest antioxidant activity (248.56 ± 1.09 μ M Fe^{2+} /g). *B. persicum* extract and *E. globulus* essential oil were the least antioxidant activity. Overall, the total antioxidant activity ranked as follows: *Bunium persicum* essential oil > *Eucalyptus globulus*

Table 2. The Chemical Components of *Bunium persicum*, *Eucalyptus globulus* Essential Oils, and Rose Water.

Plants	Compounds
<i>Bunium persicum</i> essential oil	β -pinene (11.72%), p-cymene (15.47%), γ -terpinene (18.32%), cumin aldehyde (38.4%), p-mentha-1,3-dien-7-al (5.37%), and p-mentha-1,4-dien-7-al (2.86%)
<i>Eucalyptus globulus</i> essential oil	Limonene (9.4%) and 1,8-cineole (70.3%)
Rose water	Linalool (6.6%), terpineol (3.3%), carvone (0.31%), citronellol (6.85%), trans-geraniol (7.11%), phenylethanol (66.84%), eugenol (4.53%), cytronellol, hydroxyl (1.15%), and geranic acid (1.2%)

**Figure 1.** Antioxidant activity determined by the ferric reducing antioxidant power assay.**Table 3.** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the *Bunium persicum*, *Eucalyptus globulus*, and Rose Water Against *Listeria* spp Isolated From Seafood.

Name of Medicinal Plants		<i>Listeria monocytogenes</i>	<i>Listeria grayi</i>
MIC (mg/mL)	<i>Bunium persicum</i> EO	0.351	2.812
	<i>Eucalyptus globulus</i> EO	0.351	1.406
	Rose water	NE	NE
	<i>Bunium persicum</i> E	247	495
	<i>Eucalyptus globulus</i> E	45	361
MBC (mg/mL)	<i>Bunium persicum</i> EO	0.703	5.625
	<i>Eucalyptus globulus</i> EO	0.703	2.812
	Rose water	NE	NE
	<i>Bunium persicum</i> E	495	990
	<i>Eucalyptus globulus</i> E	90	723

Abbreviations: E, ethanolic extract; EO, essential oil; NE, no effect.

extract > rose water > *Bunium persicum* extract \geq *Eucalyptus globulus* essential oil.

The results related to the MIC and MBC of the essential oils and extracts by microdilution method are given in Table 3. The essential oil of *E. globulus* showed consistently strong

antimicrobial activity against *L. monocytogenes* and *L. grayi*, while rose water showed no antimicrobial activity against any of the tested bacterial strains. The essential oils generally exhibited a higher antimicrobial spectrum than extracts. Interestingly, *L. monocytogenes* was more sensitive to essential oils and extracts than *L. grayi*.

Table 4 shows the results when *Listeria* strains were treated with MIC concentration of essential oils and extracts for 4 hours, respectively. The results showed that after adding the *B. persicum* and *E. globulus* essential oils to bacteria, the cell components' release increased significantly. Compared with control and the cell components in suspensions treated with MIC concentration of *B. persicum* and *E. globulus* essential oils for *L. monocytogenes* increased by 2.52 and 2.86 times, respectively and they increased by 1.52 and 1.67 times, respectively, for *L. grayi*.

Discussion

This study showed that prevalence of *L. monocytogenes* in shrimp and fresh fish samples taken in the Kashan region, was low (0.94%). Previous studies in other areas in Iran have been demonstrated as follows; 7.8% of open-air fish market samples in Mazandaran,⁸ 0% and 1.9% of fresh fish, frozen fish, and shrimp samples in Isfahan and Shahrekord, respectively.^{10,20} Also, our findings are similar to Momtaz and Yadollahi²⁷ who revealed 2.5% (1 sample) *L. monocytogenes* contamination in fresh shrimp in Iran. Zarei et al²⁸ reported the low prevalence of *L. monocytogenes* in Iranian seafood samples (1.4%). Latorre et al²⁹ reported that *L. monocytogenes* was not found in 154 samples of fresh seafood products in Italy.

The results of this work indicate resistance of *Listeria* spp to antibiotics (Table 1). *L. monocytogenes* was resistant to ampicillin and penicillin as the first-choice antibiotics used for treatment of human listeriosis. Erythromycin and trimethoprim/sulfamethoxazole are generally used for treatment of listeriosis in pregnant women and patients having allergy to penicillin, respectively. In this study, *L. monocytogenes* was susceptible to trimethoprim/sulfamethoxazole that is in agreement with the previous reports.^{30,31} In addition, *L. monocytogenes* was resistant to vancomycin as one of the last therapeutic options for the treatment of human listeriosis.³² Lakhanpal et al³³ showed that 3 *L. monocytogenes* isolated from milk products were sensitive to ampicillin, gentamicin, vancomycin, and erythromycin. However, the isolates were resistant to nalidixic acid and ciprofloxacin. Rahimi et al³⁴ showed that 3 *L. monocytogenes* isolated from smoked and salted fish samples were resistant to nalidixic acid, penicillin, and tetracycline. Also, the isolates were sensitive to ciprofloxacin, vancomycin, gentamycin, and chloramphenicol.

Recently, due to the increasing problem of bacterial resistance, extracts and essential oils are regarded as good candidates for replacing chemical antimicrobial agents. Previous works have demonstrated the antioxidant activity and antibacterial properties of essential oils and extracts.^{2,23,25,26}

Table 4. Effects of *Bunium persicum*, *Eucalyptus globulus*, and Rose Water in MIC Concentrations on Cell Constituents' Release of *Listeria* spp Isolated From Seafood.*

Medicinal Plants		<i>Bunium persicum</i> EO	<i>Eucalyptus globulus</i> EO	Rose water	<i>Bunium persicum</i> E	<i>Eucalyptus globulus</i> E
Cell constituents (OD _{260 nm})	<i>Listeria monocytogenes</i>	0.136 ± 0.001 ^a	0.675 ± 0.002 ^a	—	ND	ND
	Control	0.054 ± 0.000 ^b	0.236 ± 0.000 ^b	—	ND	ND
	<i>Listeria grayi</i>	0.149 ± 0.001 ^c	0.797 ± 0.001 ^c	—	ND	ND
	Control	0.098 ± 0.000 ^d	0.476 ± 0.000 ^d	—	ND	ND

E, ethanolic extract; EO: essential oil; ND, not detected; OD_{260 nm}, optical density at 260 nm.

*Different superscript letters a, b, c, and d in the column indicate significant differences ($P = .00$).

Our results showed that *B persicum*, *E globulus*, and rose water had antioxidant activities (Figure 1), which was supported by previous studies.³⁵⁻³⁷ Rose water is called “Golab” in Iran. It has been used to prepare drinks and as a traditional medicine for several centuries in Iran.³⁵ Ganesan et al³⁸ reported that the phenolic compounds isolated from *E globulus* demonstrated antioxidant activity and antioxidative property against glucose- and oxalate-induced oxidative stress in NRK-49F cells. El-Sayed et al³⁹ demonstrated that the Taif rose water byproduct had free radical scavenging activity toward artificial 1,1-diphenyl picrylhydrazyl radical with $SC_{50} = 23.72 \pm 0.36 \mu\text{g/mL}$. The results in this work indicate that these plants could be a potential sources of natural antioxidant foods.

The 4 essential oils and extracts inhibited the growth of *L monocytogenes* and *L grayi*. In the present work, 2 essential oils studied had a lower MIC and MBC when used against the Gram-positive *L monocytogenes* and *L grayi*. Our results are in agreement with the previous studies.^{40,41} Patra and Beak⁴¹ have reported the *Enteromorpha linza*, *Undaria pinnatifida*, *Laminaria japonica*, and *Porphyra tenera* essential oils exhibited strong antilisterial activity against multiple strains of *L monocytogenes*. A previous study has demonstrated antibacterial activity of cumin and eucalyptus essential oils against *L monocytogenes*, *Salmonella typhi*, *Streptococcus pyogenes*, and *Shigella dysenteriae*. *L monocytogenes* has the same sensitivity to cumin and eucalyptus essential oils. The MIC and MBC values were 5.625 and 11.25 mg/mL, respectively.⁴²

The difference antimicrobial properties of extracts and essential oils against *Listeria* spp may be linked to inoculation amount of bacteria, incubation time as experimental conditions, and the sources of extracts and essential oils and their chemical composition.⁴⁰

The results of this study showed, increase in the optical density values at 260 nm of *L monocytogenes* and *L grayi* treated with *B persicum* and *E globulus* essential oils over time, which can result in changes of cell membrane structure. Also, the cellular leakage can result from the effect of penetration of the hydrophobic essential oils. Therefore, some reducing sugars, K^+ , Ca^{2+} , and Na^+ as small molecules or macromolecules, including proteins and nucleic acids released from the bacteria to the outside. Thus, we concluded that one of the modes of antibacterial action on *Listeria* spp was that the essential oils from *B persicum* and *E globulus* first destroyed the cell

membranes, next causing the leakage of cytosolic materials, which finally resulted in the cell death.

Similar observations regarding the release of cytosolic materials outside of bacterial cells treated with essential oils from *Enteromorpha linza*, *Undaria pinnatifida*, *Laminaria japonica*, *Porphyra tenera*, *Laurus nobilis*, *Mentha pulegium*, *Satureja calamintha*, *Lavandula stoechas*, and *Myrtus communis* and other essential oils have been reported.^{26,40,41}

Conclusion

Considering the low prevalence of *L monocytogenes* in this study, consumption of raw and undercooked seafoods, may pose a health risk particularly for susceptible populations.

The present work confirmed the antioxidant activity of the *B persicum*, *E globulus*, and rose water. Also, *B persicum* and *E globulus* essential oils and extracts had relatively high antimicrobial activity against *Listeria* spp. In addition, the essential oils can penetrate the cytoplasmic membrane, which causes a loss of the membrane permeability of the cellular membrane and leakage of the cytosolic materials. Therefore, it is suggested that the above extracts and essential oils used as a potential source of natural antibacterial agents against multidrug-resistant *Listeria*.

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Author Contributions

FSC contributed to the design of the study, supervised the work scientifically, and edited the English manuscript. MS contributed to the data collection and laboratory testing. RSC developed the original idea, analyzed and abstracted the data, and prepared the manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The study protocol was approved by Ethical Committee of Kashan University of Medical Sciences, Kashan, Iran.

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